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Srivastava, K., & Bayraktutan, U. (2017). Suppression of Protein Kinase C- α Ameliorates Hyperglycaemia-Evoked In Vitro Cerebral Barrier Dysfunction. *Stroke Research and Therapy*, 2(1).
<http://stroke.imedpub.com/abstract/suppression-of-protein-kinase-c-ameliorates-hyperglycaemiaevoked-in-vitro-cerebral-barrier-dysfunction-19255.html>

Published in:
Stroke Research and Therapy

Document Version:
Publisher's PDF, also known as Version of record

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Suppression of Protein Kinase C- α Ameliorates Hyperglycaemia-Evoked *In Vitro* Cerebral Barrier Dysfunction

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Abstract

Title: Suppression of protein kinase C- α ameliorates hyperglycaemia-evoked *in vitro* cerebral barrier dysfunction.

Background: Ischaemic stroke is common in diabetes patients and occurs due to cerebral-barrier dysfunction. Although the pathological activation of protein kinase C (PKC) is linked with the disease progression, the PKC isoform-specific downstream signalling remains obscure and is the focus of this study which explored the clinical relevance of PKC- α inhibitor Ro-32-0432 under hyperglycaemia (HG).

Methods and Findings: Total PKC and RhoA activities were studied in human brain microvascular endothelial cells (HBMEC) exposed to normoglycaemia or HG. The integrity and function of an *in vitro* model of human BBB composed of HBMEC and human astrocytes were measured by transendothelial electrical resistance (TEER) and paracellular flux of Evans blue-labelled albumin (EBA), respectively. Exposure of HBMEC to HG for 72 hours led to significant increases in activities of total PKC and RhoA as well as in mono- and di-phosphorylation of MLC2 which concurred with substantial decreases in TEER and marked elevations in barrier permeability. Enhanced cellular contractility triggered by actin stress fibre formation appeared to further potentiate HG-mediated barrier dysfunction. Pre-treatment of HBMEC with Ro-32-0432 or transient PKC- α protein knockdown led to effective preservation of BBB integrity and function in hyperglycaemic settings by suppressing RhoA activity and subsequently normalising the MLC2 phosphorylation on Ser19 and Thr18-Ser19 residues. These observations were further supported by disappearance of stress fibres and subsequent restoration of the cortical actin staining.

Conclusions: Neutralisation of PKC- α activity may be of considerable therapeutic value in clinical settings accompanied by diabetes or stress HG.

Keywords: Endothelial cell; Hyperglycaemia; Protein kinase C; Ro-32-0432; RhoA; cytoskeletal remodeling

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Citation: Srivastava K, Bayraktutan U. Suppression of Protein Kinase C- α Ameliorates Hyperglycaemia-Evoked *In Vitro* Cerebral Barrier Dysfunction. *Stroke Res Ther.* 2017, 2:1.

Received: March 20, 2017; **Accepted:** April 13, 2017; **Published:** April 18, 2017

Introduction

The vascular endothelium lines the entire inner surface of all blood vessels and forms specific barriers in different organs to regulate vascular permeability. The blood-brain barrier (BBB) represents one such barrier and prevents the leakage of blood-borne substances into brain parenchyma. Disruption of the BBB characterised by the formation of brain oedema is a common occurrence after ischaemic strokes and constitutes the main cause of mortality within the first week after a cerebrovascular

attack. As the brain oedema is more prevalent in stroke patients with diabetes mellitus, it is thought that hyperglycaemia may be intricately involved in the pathogenesis of this defect [1-3].

The BBB consists of brain microvascular endothelial cells (BMEC), capillary basement membrane and astrocyte end-feet supporting the basement membrane. The permeability of the BBB is tightly regulated by tight junction proteins, notably occludin and claudin-5, that exist between the adjacent BMEC. Hence, pathologies that affect BMEC phenotype and/or tight junction protein expression and localisation would have an adverse

effect on the integrity and function of the BBB. Although several biochemical pathways including non-enzymatic glycation of proteins and lipids with the irreversible deposition of advanced glycation end products, the activation of polyol pathway or stimulation of protein kinase C (PKC) pathway have been implicated in diabetes-mediated peripheral vasculopathies, the mechanisms involved in hyperglycaemia-evoked cerebrovascular impairments remain largely unknown [4].

In addition to regulation of PKC activity, the suppression of RhoA may also be an efficacious therapeutic option in protecting cerebral barrier integrity given the fact that binding of active RhoA to its downstream effector Rho-kinase leads to the disruption of intercellular junctions by sequential induction of myosin-regulatory light chain-2 phosphorylation (pMLC2) and actin stress fibre formation [5-16].

In light of the above, this study investigated the specific role of PKC- α isoform in hyperglycaemia-mediated cerebral damage using an *in vitro* model of human BBB. In addition, comprehensive analyses of the relationship between PKC- α isoform and RhoA/pMLC2 pathway were performed to unravel new therapeutic targets that can be utilised to prevent or minimise cerebral barrier damage in hyperglycaemic settings.

Material and Methods

Cell culture

Human BMEC (HBMEC) and astrocytes (HA) were purchased from TCS CellWorks, UK and cultured up to passage 6 in their respective specialised medium in humidified atmosphere under normal conditions (70% N₂, 25% O₂ and 5% CO₂ at 37°C) before exposure to normoglycaemia (5.5 mM D-glucose) or hyperglycaemia (25 mM D-glucose) for 72 hours. The significance of changes in PKC- α activity to HG-mediated endothelial-barrier damage was assessed using a specific inhibitor, Ro-32-0432 (1-5 μ M). In a subset of experiments, HBMEC were initially cultured in hyperglycaemic conditions for 72 hours before a further 72 hours culture in normoglycaemic conditions to assess the specific effects of glucose normalisation (glycaemic control). Cells were also cultured in equimolar concentrations of D-mannitol (25 mM) to assess if the changes observed in enzyme activities and BBB integrity were due to hyperosmolality rather than HG per se.

In vitro model of human BBB

HA were seeded on the outside of polyester membrane (0.4 μ m pore size) transwell inserts (Corning Costar, High Wycombe, UK) directed upside down in the 6-well tissue culture plates. Following the overnight adherence of HA to the membranes, the inserts were inverted the correct way in the 12-well tissue culture plates and HBMEC were seeded onto the inner side of the polyester membrane. The cells were cultured to confluence before exposing co-cultures to the experimental conditions.

BBB experiments

The integrity and function of human BBB were assessed by measurements of transendothelial electrical resistance (TEER) and the flux of Evan's blue-labelled albumin (EBA), a high

molecular weight permeability marker (67 kDa), across the co-cultures as previously described [17].

Immunoblotting

To evaluate the fold differences in protein levels, immunoblottings were performed [15] using the specific antibodies raised against total RhoA, total MLC2, pMLC2-Ser19, pMLC2-Thr18-Ser19, PKC- α , PKC- β (Santa Cruz Biotech) and β -actin (Sigma-Aldrich, UK).

Immunofluorescence

HBMEC cultured on 8-chamber tissue slides (Lab-Tek) were fixed and permeabilised with 4% paraformaldehyde/PBS and 0.1% Triton X-100/PBS, respectively for 20 min each before visualising actin cytoskeleton via Rhodamine-labelled phalloidin dye (5 U/mL, Invitrogen, UK). The cells were stained with 4,6-diamidino-2-phenylindole to visualise the nuclei and viewed under 40X magnification on a Zeiss Axio Observer fluorescence microscope (Germany).

Rho activity assay

The RhoA activity was measured using a "pull-down" assay kit (Millipore, UK). Briefly, about 150 μ g of HBMEC lysates were incubated with Rhotekin Rho-binding peptide immobilized on agarose beads (10 μ g) prior to detection of activated guanosine triphosphate-RhoA bound to Rhotekin by immunoblotting.

PKC activity assay

Total PKC activity was determined in HBMEC lysates as previously described using the non-radioactive PKC activity assay kit (Promega, UK) which utilises a brightly coloured PKC-specific peptide substrate, PepTag C1 [17].

Transient knockdown

To knockdown PKC- α protein expression, semi-confluent HBMEC were transfected for 24 hours with DharmaFECT siRNA transfection reagent-4 containing 50 nM of either ON-TARGET plus non-targeting pool of small interfering RNA (siRNA) or ON-TARGET plus SMART pool human siRNA against PKC- α (GE Dharmacon). Following first round of transfection, the cells were allowed to recover in the fresh medium for 6 hours before second round of transfection with 50 nM of aforementioned siRNA molecules. The cells were homogenised after 72 hours to assess the downregulation of protein expression by immunoblotting.

Assessment of cell viability

A fraction of cells exposed to different treatments were incubated with 0.1% trypan blue for 5 minutes and the percentages of cell viability were calculated by counting 100 cells on a haemocytometer under light microscope.

Statistical Analysis

Statistical analyses were performed using IBM SPSS statistics 21.0 software package. The mean values were compared by Student's two-tailed t-test or one-way ANOVA, where appropriate, followed by Dunnet's post-hoc analysis. P<0.05 was considered significant.

Results

Inhibition of PKC- α protects BBB integrity and function under hyperglycaemic conditions

Using an *in vitro* model of human BBB established by co-culture of HBMEC and HA, we have recently shown that inhibition of total PKC with Bis-I negates the deleterious effects of hyperglycaemia on BBB integrity [14]. The present study shows that specific attenuation of PKC- α activity by Ro-32-0432 also normalises the disruptive effects of hyperglycaemia on cerebral barrier structure and function (**Figure 1A-B**).

PKC- α modulates the activation of RhoA signalling in hyperglycaemic settings

Since overactivities of PKC and RhoA have previously been implicated in cerebral barrier dysfunction [15,17-19] and Ro-32-0432 effectively maintains barrier integrity and function in hyperglycaemic settings, it is possible that inhibition of PKC- α may protect BBB by concomitantly regulating the activities of total PKC and RhoA. Indeed, inhibition of PKC- α reduces the total PKC activity in HBMEC below the levels seen in cells exposed to equal periods of hyperglycaemia, normoglycaemia or hyperglycaemia followed by normoglycaemia (**Figure 2**). Moreover, inhibition of PKC- α normalised the hyperglycaemia-mediated increases observed in RhoA-GTP protein levels, a measure of RhoA activity without affecting that of total RhoA (**Figure 3A**). In support of this, attenuation of PKC- α gene expression by a specific siRNA appeared to diminish RhoA-GTP protein levels without affecting total RhoA levels in HBMEC exposed to normoglycaemia and hyperglycaemia. In these experiments, suppression of the basal RhoA activity in normoglycaemic cells by PKC- α siRNA confirmed the efficacy of this experimental procedure (**Figure 3B**). Moreover, the inefficacy of PKC- α siRNA to affect PKC- β protein expression confirmed the specificity of the siRNAs employed for PKC- α (**Figure 3C,D**).

PKC- α mediates hyperglycaemia-evoked cytoskeletal remodelling

Cytoskeletal reorganisation induced by activation of RhoA has previously been coupled to enhanced cerebral barrier permeability [15,17,19]. To evaluate whether barrier-protective effects of PKC- α inhibition may in part be attributed to its modulatory effects on endothelial cell cytoskeletal proteins MLC2 and F-actin, this study examined the effects of hyperglycaemia on MLC2 mono- and di-phosphorylation levels and F-actin cellular localisation in the absence or presence of Ro-32-0432. While hyperglycaemia alone evoked a dramatic increase in the protein levels of pMLC2-Ser19 and pMLC2-Thr18/Ser19, co-treatment with Ro-32-0432 suppressed both phosphorylation rates in a dose-dependent manner without altering the total MLC2 levels (**Figure 4A-B**). In addition to this, hyperglycaemia promoted the formation of stress fibres traversing the cells. However, co-treatments with Ro-32-0432 eradicated stress fibres and restored cortical actin localisation similar to those seen in normoglycaemic cells (**Figure 4C**). Silencing of PKC- α expression in normoglycaemic and hyperglycaemic HBMEC via siRNA technology fully corroborated these results (**Figure 5A-C**).

Effects of glucose normalisation and osmolality on enzyme activities and BBB integrity

Hyperglycaemia-induced increases observed in total RhoA activity and impairments in BBB integrity and function were independent of a rise in osmolality as ascertained by application of equal concentrations of D-mannitol (25 mM) and were completely neutralised by normalisation of glucose levels (**Table 1**).

Discussion

hyperglycaemia stemming from an imbalance in glucose metabolism adversely affects vascular homeostasis and exacerbates the intensity of vasculopathies, notably endothelial dysfunction commonly encountered in patients with history

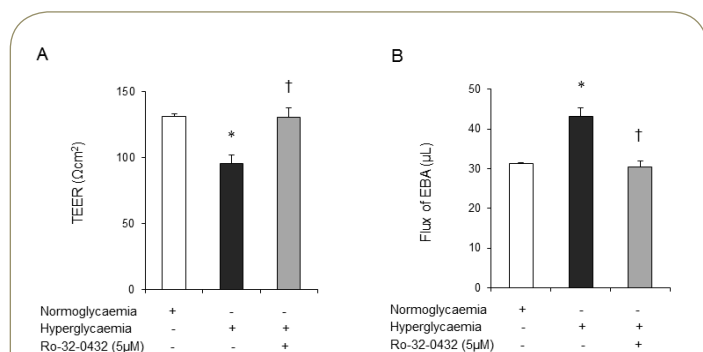


Figure 1 Effects of normoglycaemia (D-glucose 5.5mM) and hyperglycaemia (D-glucose 25 mM) on an *in vitro* model of human blood-brain barrier integrity and function in the absence or presence of a specific inhibitor for PKC- α , Ro-32-0432 (5 μ M), as assessed by measurements of transendothelial electrical resistance (TEER) (A) and EBA flux (B), respectively. Data are expressed as mean \pm SEM from 4 independent experiments. *P<0.05 compared to normoglycaemia, †P<0.05 compared to hyperglycaemia.

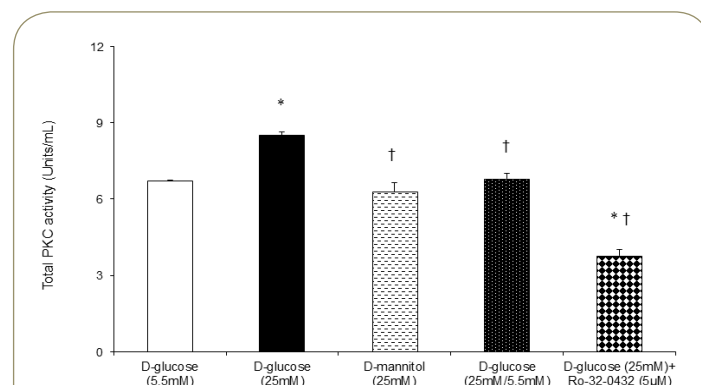


Figure 2 Effects of normoglycaemia (D-glucose 5.5 mM), hyperglycaemia (D-glucose 25 mM), osmolality (D-mannitol 25 mM), normalisation of glucose levels after hyperglycaemia and hyperglycaemia with Ro-32-0432 (5 μ M) on total protein kinase C (PKC) activity (Units/mL) in human brain microvascular endothelial. Mean \pm SEM from 4 independent experiments. *P<0.05 compared to normoglycaemia, †P<0.05 compared to hyperglycaemia.

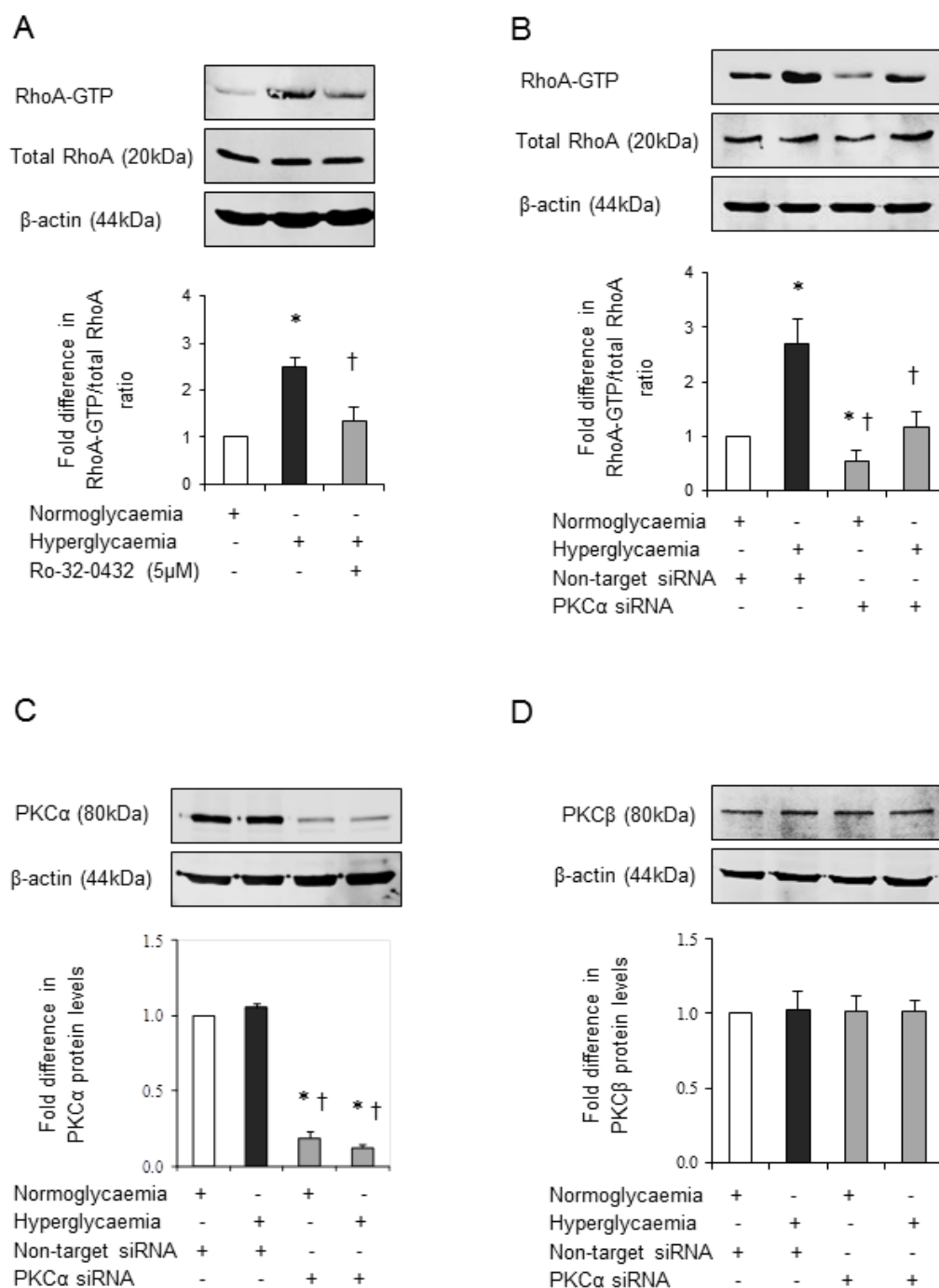


Figure 3 Effects of normoglycaemia (D-glucose 5.5 mM) and hyperglycaemia (D-glucose 25 mM) alone or in the presence of a PKC- α inhibitor Ro-32-0432 (5 μ M) on total RhoA activity (A). Effects of non-targeting and PKC- α specific siRNA molecules on the total RhoA activity (B), protein expressions of PKC- α (C), PKC- β (D) in human brain microvascular endothelial cells exposed to normoglycaemia or hyperglycaemia. Data are expressed as mean \pm SEM from 3 independent experiments. * P <0.05 compared to normoglycaemia, $^{\dagger}P$ <0.05 compared to hyperglycaemia.

of myocardial infarction and stroke. Although endothelial dysfunction in cerebral vasculature is intimately associated with the initiation and/or progression of BBB failure, the molecular mechanism(s) involved remains largely unknown. Using an *in vitro* model of human BBB composed of HBMEC and astrocytes, the current study reveals PKC- α as an important component in

hyperglycaemia-mediated BBB dysfunction. Activation of PKC- α has been shown to enhance the levels of small GTP-binding protein, RhoA which in turn successively modulates phosphorylation and localisation of cytoskeletal proteins, namely MLC2 and actin microfilaments and as a consequence promote cytoskeletal reorganisation and BBB leakage. Inhibition of PKC- α activity under

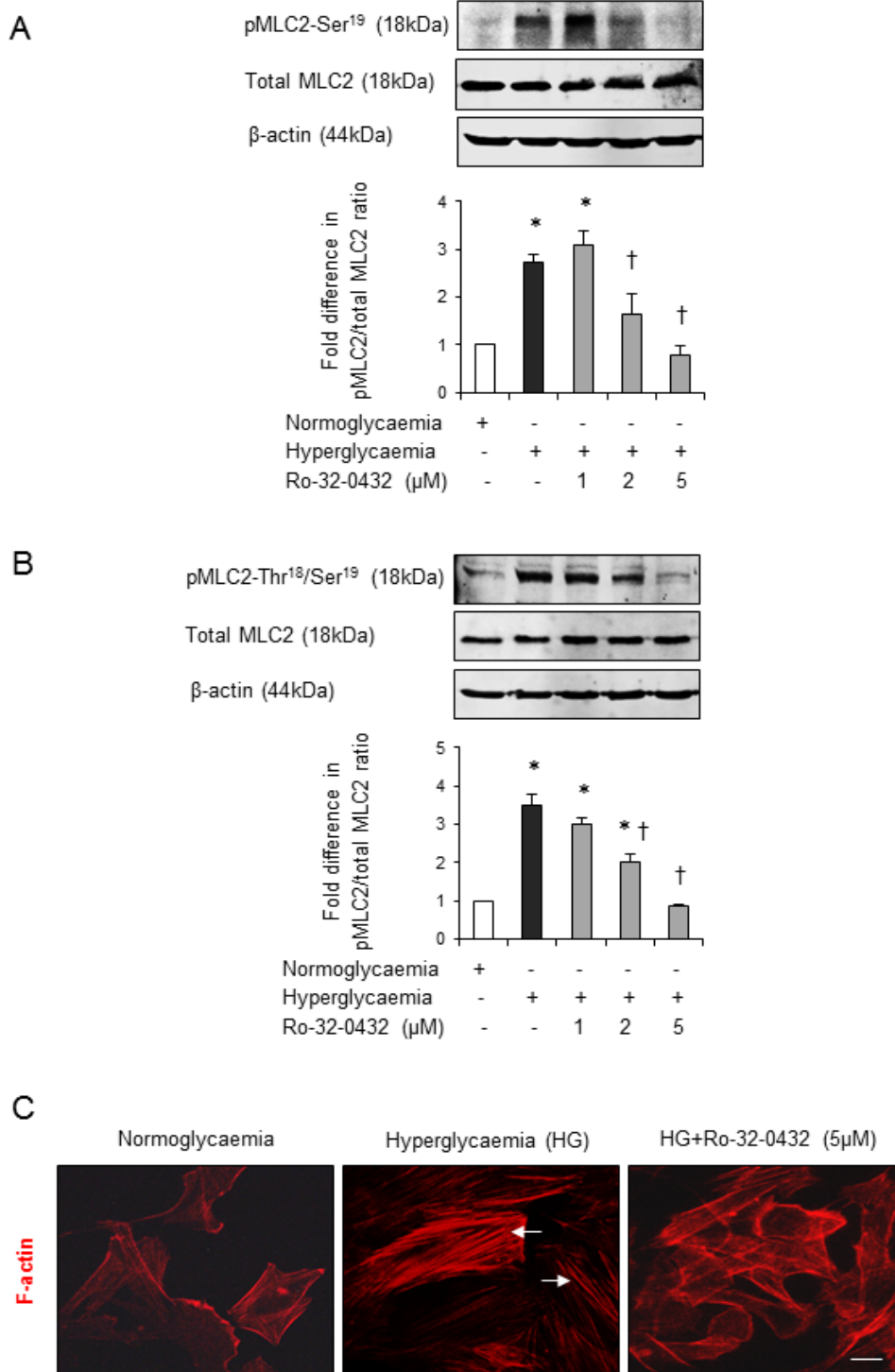


Figure 4 Dose-dependent effect of Ro-32-0432 on MLC2 mono-phosphorylation (A), di-phosphorylation (B) and F-actin staining (C) in human brain microvascular endothelial cells exposed to normoglycaemia (D-glucose 5.5mM) or hyperglycaemia (D-glucose 25mM) with/without PKC- α inhibitor Ro-32-0432 (5 μ M). Arrows indicate stress fibre formations. Scale bar: 50 μ m. Data are expressed as mean \pm SEM from 3 independent experiments. * P <0.05 compared to normoglycaemia, † P <0.05 compared to hyperglycaemia.

hyperglycaemic conditions by Ro-32-0432 or PKC- α siRNA appear to alleviate cytoskeletal remodelling and improve endothelial barrier integrity and function by normalising the RhoA activity. Repetition of similar experiments with equimolar concentration of D-mannitol (25 mM) overtly indicates that these changes were

independent of an increase in osmolality, in keeping with the findings of our previous studies [14,17]. In addition, normalisation of glucose levels after a period of hyperglycaemic insult (72 hours) appeared to mitigate the effects of hyperglycaemia on total PKC and RhoA activities and on cerebral-barrier integrity, indicating

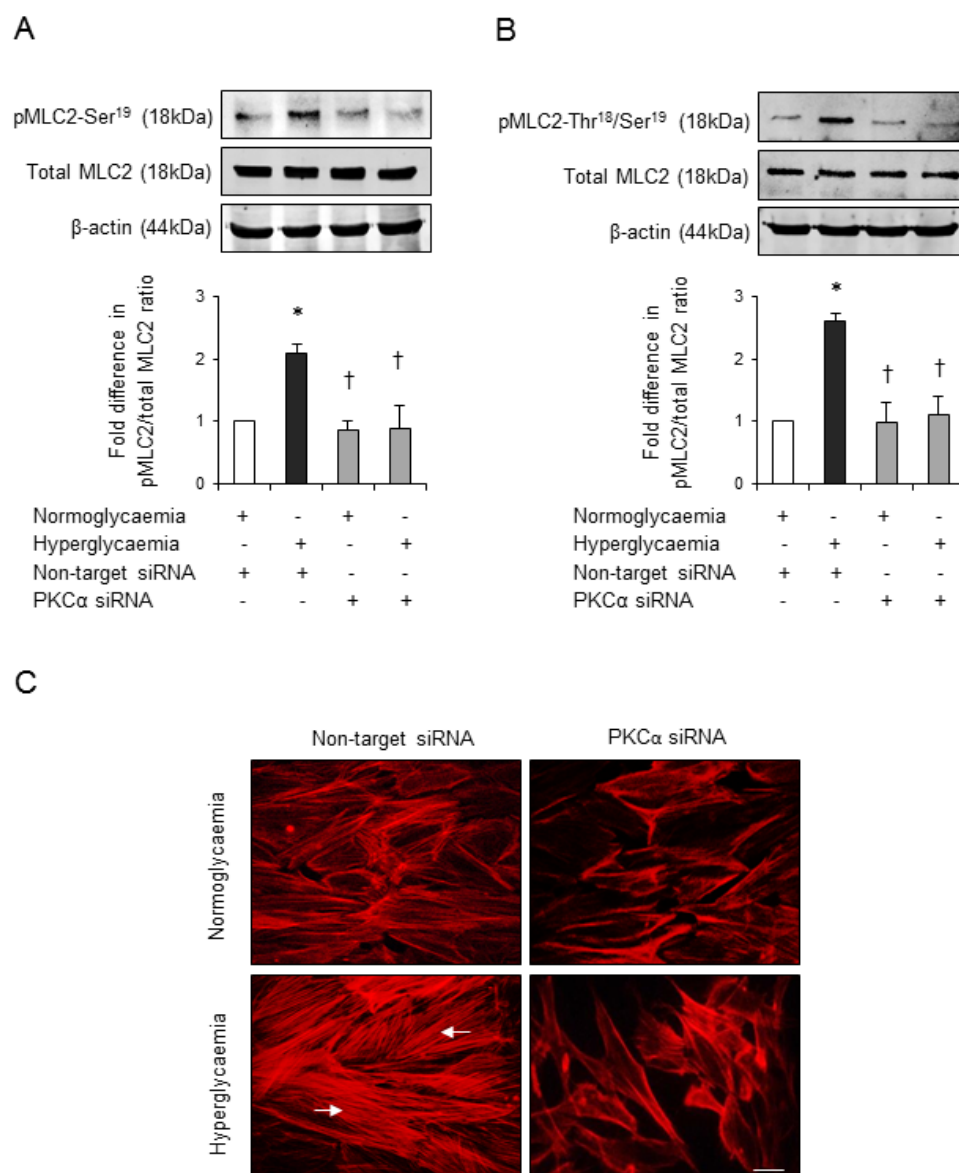


Figure 5 Effects of non-targeting and PKC-α specific siRNA molecules on MLC2 mono-phosphorylation (A), di-phosphorylation (B) and F-actin staining (C) in human brain microvascular endothelial cells exposed to normoglycaemia (D-glucose 5.5 mM) or hyperglycaemia (D-glucose 25mM). Arrows indicate stress fibre formations. Scale bar: 50 μm. Data are expressed as mean ± SEM from 3 independent experiments. *P<0.05 compared to normoglycaemia, †P<0.05 compared to hyperglycaemia.

the importance of effective glycaemic control. Indeed, while poor glycaemic control in patients with type 2 diabetes has been positively correlated with an elevated plasma level of vascular endothelial growth factor and development of diabetic retinopathy, its effective regulation, confirmed by decreases in glycated haemoglobin (HbA1c) levels, appeared to prevent the progression of diabetic retinopathy [20]. Similarly, efficacious management of hyperglycaemia in streptozotocin-diabetes rats has been shown to enhance the expression of an endoprotective transcription factor, Kruppel-like factor 2 and attenuate proteinuria, glomerular hypertrophy and endothelial injury, hallmarks of diabetic nephropathy [21].

Considering that PKC regulates a wide range of activities individually or collectively associated with endothelial

(dys) function like excessive production of reactive oxygen species, cytokines, growth factors and the activation of matrix metalloproteinases [5], it was of no surprise that treatments with Ro-32-0432 (1-5 μM), an inhibitor of PKC-α restored BBB integrity and function under hyperglycaemic conditions in a dose-dependent manner. The concentrations of Ro-32-0432 employed in this study were in the previously reported working range (1-10 μM) and did not markedly affect HBMEC viability similar to human lung epithelial cells, platelets and monocytes [22-24]. As 5 μM of Ro-32-0432 diminished RhoA-GTP activity to the levels obtained by silencing of PKC-α gene via its siRNA, this dose was used during the rest of the study.

Exposure of HBMEC to hyperglycaemia potentiated the activity of total PKC which was reduced by co-treatment with Ro-32-0432

| Treatment groups | Total RhoA activity (AU) | TEER (Ωcm^2) | EBA flux (μL) |
|------------------------|--------------------------|------------------------------|----------------------------|
| D-glucose (5.5mM) | 1.0 \pm 0.00 | 125.7 \pm 10 | 30.3 \pm 6.5 |
| D-glucose (25mM) | 3.0 \pm 0.40* | 82.6 \pm 5.5* | 47 \pm 5.5* |
| D-mannitol (25mM) | 1.3 \pm 0.20† | 128 \pm 3.5† | 28.5 \pm 3.9† |
| D-glucose (25mM/5.5mM) | 1.5 \pm 0.45† | 115.5 \pm 8.2† | 31.2 \pm 5.5† |

Mean \pm SEM from 4 independent experiments. * P <0.05 compared to normoglycaemia, † P <0.05 compared to hyperglycaemia. BBB=Blood-brain barrier, PKC= protein kinase C, AU= Arbitrary unit, TEER= Transendothelial electrical resistance, EBA= Evan's blue-labelled albumin.

Table 1: Hyperglycaemia-induced increases observed in total RhoA activity and impairments in BBB integrity and function were independent of a rise in osmolality as ascertained by application of equal concentrations of D-mannitol (25 mM) and were completely neutralised by normalisation of glucose levels.

below the levels observed in normoglycaemic cells, implying effective neutralisation of both basal and induced levels of PKC- α activity with this particular inhibitor. These results suggest that activation of PKC- α significantly contributes to the enhanced overall PKC activity and ensuing vascular pathologies in hyperglycaemic conditions. Indeed, activation of PKC- α in diabetic rats has been shown to enhance the NADPH oxidase-mediated superoxide production in renal tissues and elevate the renal, serum, and urinary vascular endothelial growth factor concentrations leading to diabetic nephropathy [25]. Similarly, activation of PKC- α has been coupled to increased incidence of heart failure in mice where its effective inhibition improved ventricular performance and reduced fibrosis and pulmonary oedema [26].

Similar to total PKC, exposure of HBMEC to hyperglycaemia also resulted in an increase in RhoA activity. Like PKC, small GTPase RhoA is also implicated in various processes that can radically affect the integrity and permeability of the BBB. Once activated, RhoA has been shown to bind to its effector protein Rho-kinase and promote stress fibre formation and MLC2 phosphorylation which in turn increase paracellular flux by destabilising cell junctions thereby suggesting the notion that inhibition of RhoA may be of paramount importance in attaining normal microvascular function and negating barrier permeability [15,27,28]. Indeed, inhibition of Rho-kinase activity by Y-27632 has been shown to completely abolish these effects

in different conditions known to affect BBB integrity including hyperglycaemia and oxygen-glucose deprivation alone or followed by reperfusion [15,17]. In accordance with these findings, in the current study hyperglycaemia has been shown to trigger MLC2 phosphorylation and cytoskeletal reorganisation characterised by appearance of thick actin stress fibres traversing the cells. Interestingly, inhibition of PKC- α activity by Ro-32-0432 in HBMEC mimicked the endothelial-barrier protective effects of Y-27632 by normalising the RhoA activity, mono- and di-phosphorylation of MLC2, reducing stress fibre formations and restoring the F-actin localisation to the cellular cortex [17]. These results indicate that PKC- α can effectively modulate the RhoA/Rho-kinase signalling pathway. The importance of this pathway has been exemplified in a recent study with retinal pigment epithelial cells where PKC- α overactivity is associated with enhanced cell migration triggered by the formation of stress fibres and actin cytoskeletal remodelling contributing to the development of proliferative eye diseases [29]. Furthermore, *in vivo* studies with mice and *in vitro* studies with human pulmonary artery endothelial cells have also shown that activation of PKC- α with microtubule-disrupting agents like 2-methoxyestradiol increase endothelial-barrier permeability by phosphorylating ezrin, radixin and moesin proteins, known to activate the Rho signalling and promote *de novo* assembly of F-actin [30-32].

In addition to its effects on cellular architecture, activation of RhoA also affects localisation of occludin and zonula occludens-1,

tight junction proteins that prevent paracellular leakage [17]. Besides, RhoA activation potentiates the activities of matrix metalloproteinases which digest extracellular matrix components and thus enhance endothelial-barrier permeability [33]. Since Ro-32-0432 may also interfere with the activities of PKC- β [23,34], the specific relevance of PKC- α to RhoA activity, MLC2 phosphorylation and cytoskeletal remodelling in hyperglycaemic settings was investigated in HBMEC after silencing its gene expression by siRNAs which had no effect on PKC- α protein expression thereby confirming specificity. The results generated were similar to those obtained with Ro-32-0432, confirming the regulatory role of PKC- α in RhoA activation and supporting the previous studies showing PKC- α -mediated activation of RhoA at cell membrane by either regulating its dissociation from Rho guanine dissociation inhibitor 1-RhoA complex to promote its cytoplasmic trafficking as seen in pancreatic acini or by phosphorylating p115Rho guanine exchange factor to facilitate the exchange of GDP with GTP as reported in mouse BMEC [35]. Along with promoting cytoskeletal remodelling, enhanced PKC- α activity may also compromise endothelial barrier integrity by inducing apoptosis, matrix metalloproteinase activity or oxidative stress through modulating the excessive synthesis of reactive oxygen species, in particular, superoxide anion by pro-oxidant enzyme NADPH oxidase and/or suppressing nitric oxide availability, a key regulator of vascular homeostasis by uncoupling endothelial nitric oxide synthase (eNOS) [36,37]. Hence, selective inhibition of conventional PKC isoforms in type 2 diabetic rats by Ro-32-0432 and Go-6976 have been shown to mitigate oxidative stress in conduit arteries by both restoring eNOS function and attenuating NADPH oxidase hyperactivity [38,39]. Furthermore, siRNA-mediated inhibition of PKC- α in HBMEC neutralised the barrier-disruptive effects of oxygen-glucose deprivation and TNF- α , an inflammatory cytokine by attenuating oxidative stress, apoptosis and the activity of basement membrane-degrading enzymes [40].

It is noteworthy here that both normalisation of glucose levels and

targeted silencing the activities of other conventional isoforms, namely PKC- β , PKC- β I or PKC- β II have previously been shown to improve *in vitro* BBB integrity and function in hyperglycaemic settings by negating the stimulatory effects of hyperglycaemia on endothelial cell apoptosis, cytoskeletal remodelling, oxidative stress, NADPH oxidase and MMPs activities and RhoA/Rho-kinase/MLC2 expression, activity and localisation. Inhibition of PKC- α in these studies has also been shown to attenuate apoptosis and NADPH oxidase activation in hyperglycaemic HBMEC [17,18,41].

Taken together with the current data, our results collectively indicate that activation of conventional PKC isoform i.e. PKC- α may augment the extent and severity of BBB damage in neurodegenerative conditions such as ischaemic stroke accompanied by uncontrolled diabetes or stress hyperglycaemia by concomitantly inducing various interrelated molecular mechanisms and that pharmacological inhibition of this isoform can both successfully maintain barrier function and diminish the incidence of secondary complications [42]. Bearing in mind the results of our previous studies and the necessity to develop efficacious therapeutic regimens that can be used as alternatives to the currently available sole medical therapy with recombinant tissue plasminogen activator after diabetic stroke, it is important to extend the present study to a translational model in order to investigate whether simultaneous suppression of multiple conventional PKC isoforms may exert better protection on cerebral barrier as compared to inhibition of a single isoform.

Acknowledgement

The authors would like to acknowledge The University of Nottingham, UK for conducting the PhD programme in Clinical Neurosciences, which led to the preparation of this manuscript.

Funding

The authors like to acknowledge The University of Nottingham, UK for funding the study in form of PhD studentship.

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